

Research Article

Symplegma (Ascidiacea: Styelidae), a non-indigenous genus spreading within the Mediterranean Sea: taxonomy, routes and vectors

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Abstract

Symplegma is a genus of compound ascidians (Fam. Styelidae) with warm water affinities and distribution in tropical and subtropical waters of the Pacific, Indian and Atlantic Oceans. The first record of this genus (as *S. viride*) in the Mediterranean was from 1951 in the Levantine Sea, presumably entering the basin from the Red Sea through the Suez Canal. Subsequently, it has been expanding its distributional range northward along the Levantine Sea coast, probably following the prevailing surface current direction. Recently, *Symplegma* has colonized the Aegean, Ionian and Tyrrhenian Seas, where it is spreading quickly, most likely mediated by shipping (i.e., hull fouling). Some specimens from the Ionian Sea (specifically from Tunisia, Malta) present opaque tunics resembling the Indo-Pacific *Symplegma bahraini*; however, morphological studies suggest that the genus in the Mediterranean Sea is represented by a single species, *Symplegma brakenhielmi*. The taxonomy of *S. brakenhielmi*, as well as its spreading routes and possible introduction vectors are analysed.

Key words: alien species, colonial ascidians, color morphs, maritime traffic, distribution

Introduction

The opening of the Suez Canal, the expansion and increase in intensity of maritime traffic, aquaculture and the marine aquarium species trade are the main vectors of introduction of non-indigenous taxa in the Mediterranean Sea, accelerated and favoured by climatic change (Zibrowius 1992; Bianchi and Morri 2003; Streftaris et al. 2005; Galil 2006; Occhipinti-Ambrogi 2007; Abdulla and Linden 2008; Zenetos et al. 2012; Ferrario et al. 2017). Among the world's seas, the Mediterranean Sea is the most invaded by non-indigenous species (NIS), at present hosting about 700 confirmed marine NIS (Zenetos et al. 2017; Galil et al. 2018). One genus of NIS

ascidians found in the Mediterranean is *Symplegma*, which normally occurs in shallow and warm waters of tropical and subtropical seas around the world (Van Name 1945; Tokioka 1967; Monniot and Monniot 1997a; Kott 2004).

Globally, there are 11 species within the *Symplegma* genus (Sanamyan 2007). Three species [*Symplegma viride* Herdman, 1886; *Symplegma brakenhielmi* (Michaelsen, 1904); and *Symplegma rubra* Monniot C., 1972] have a circumtropical distribution in the Indo-Pacific and the Atlantic Oceans, while the other *Symplegma* species occur solely in the Indian and Pacific Oceans, some of which present a wide distribution. For instance, *Symplegma reptans* (Oka, 1927) colonizes the Western and Eastern Pacific coasts (Nishikawa 1991; Lambert and Lambert 1998, 2003), while *Symplegma bahraini* Monniot C. and Monniot F., 1997 spans the whole Indo-Pacific region (Monniot 2002; Kott 2004). Other species have been reported from more restricted areas, such the Western Pacific (*Symplegma connectens* Tokioka, 1949; *Symplegma japonica* Tokioka, 1962; *Symplegma arenosa* Kott, 1972; *Symplegma alterna* Monniot C., 1988; and *Symplegma teruakii* Kott, 2004) and Indian Ocean (*Symplegma zebra* Monniot C., 2002).

Amongst the *Symplegma* species, only *S. brakenhielmi* has been reported from the Mediterranean. The first record was made in 1951 off Cesarea (Israel) as *S. viride* by Pérès (1958a). Since then, the genus has exhibited a slow northwards expansion (Bitar and Kouli-Bitar 2001; Çinar et al. 2006; Izquierdo-Muñoz et al. 2009; Shenkar and Loya 2009). In 2008, the species was recorded in the Ionian Sea (present work), probably introduced via shipping, since it had not been previously recorded from the east coast of Greece and southern Italy, areas which would putatively have been colonised at an earlier stage considering the prevailing surface current pathway of the Eastern Mediterranean (Hamad et al. 2005). From 2014, new records of the genus have been made within the Aegean, Ionian and Tyrrhenian Seas (Ulman et al. 2017; Aydin-Onen 2018; Mastrototaro et al. 2019; present work), indicating its rapid expansion throughout the Mediterranean, and its striking colours have also attracted attention from SCUBA divers.

The aim of this study was to clarify the taxonomy and update the distribution of the *Symplegma* genus in the Mediterranean Sea. Additionally, we discuss possible temporal changes in the distribution of the genus and the likely introduction vectors for *S. brakenhielmi* in the Mediterranean Sea. A distribution of *Symplegma* spp. at the global level is also provided.

Materials and methods

A comprehensive literature search on the genus *Symplegma* was conducted, including available published scientific works, “grey literature” (scientific congresses, technical reports, student theses), and web databases (Ascidian World Database, Biodiversity Heritage Library, Google Scholar,

Scopus, GBIF). Data from personal observations was also included, and first country Mediterranean records were cited according to the first actual collection/observation date, rather than the reporting date.

Colonies of *Symplegma* from different Mediterranean localities were observed and photographed at depths ranging from 0.5 to 17 m between 2005 and 2018 from natural and artificial substrates by snorkelling or SCUBA diving in the framework of different projects and studies carried out by the authors on benthic communities. Some of these colonies, collected from Egypt, Italy, Lebanon, Malta, Tunisia and Turkey (N = 14; Supplementary material Table S1) were dissected for subsequent histological studies in order to identify the specimens based on the morphological characters.

The colonies were anaesthetised with menthol crystals, fixed with 4% formalin in seawater and preserved in 70° ethanol. Some zooids from each colony were dissected, stained with Masson's haemalum, and dehydrated in ethyl and butyl alcohols for mounting on permanent slides in Canada balsam. Taxonomic identification was made considering the original and subsequent descriptions of *Symplegma* species in literature (Van Name 1945; Tokioka 1961, 1967; Monniot 1972, 1983, 1988; Kott 1985, 2004; Nishikawa 1991; Monniot and Monniot 1997a; Rocha and Costa 2005). The specimens examined in this study were deposited at the Marine Research Center of Santa Pola (CIMAR) of the University of Alicante (Spain) with the following identification codes: Sy.br-Eg01 (from Egypt), Sy.br-It01 (from Italy), Sy.br-Le01-02 (from Lebanon), Sy.br-Mt01-02 (from Malta), Sy.br-Tu01-07 (from Tunisia) and Sy.br-Tk01 (from Turkey).

Results

Taxonomic identification

In Mediterranean waters, the colonies of *Symplegma* exhibited a great variety of colour morphs (red, rose, orange, yellow, brown, cream, white, greenish-grey) which could suggest the occurrence of more than one species. The possible candidates include pantropical species, such as *S. brakenhielmi*, *S. rubra*, and *S. viride*. Other possible candidates were also considered, such as *S. reptans* from Japan (Tokioka 1949; Nishikawa 1991), which has invaded the North-eastern Pacific American coast (Lambert and Lambert 1998, 2003), and *S. bahraini* from the Indo-Pacific region (Monniot and Monniot 1997a; Kott 2004). Morphological studies led us to identify all the analysed colonies as *S. brakenhielmi*, although some of the specimens from Tunisia and Malta present morphological characters (especially an opaque tunic) resembling *S. bahraini*.

Symplegma brakenhielmi (Michaelsen, 1904)

(Figures 1, 2)

Synonymies: *Diandrocarpa brakenhielmi* var. *typica*: Michaelsen (1904, p. 50); *D. brakenhielmi* var. *stuhlmanni* Michaelsen (1904, p. 52); *D. brakenhielmi* var. *ceylomica* Herdman (1906, p. 331);

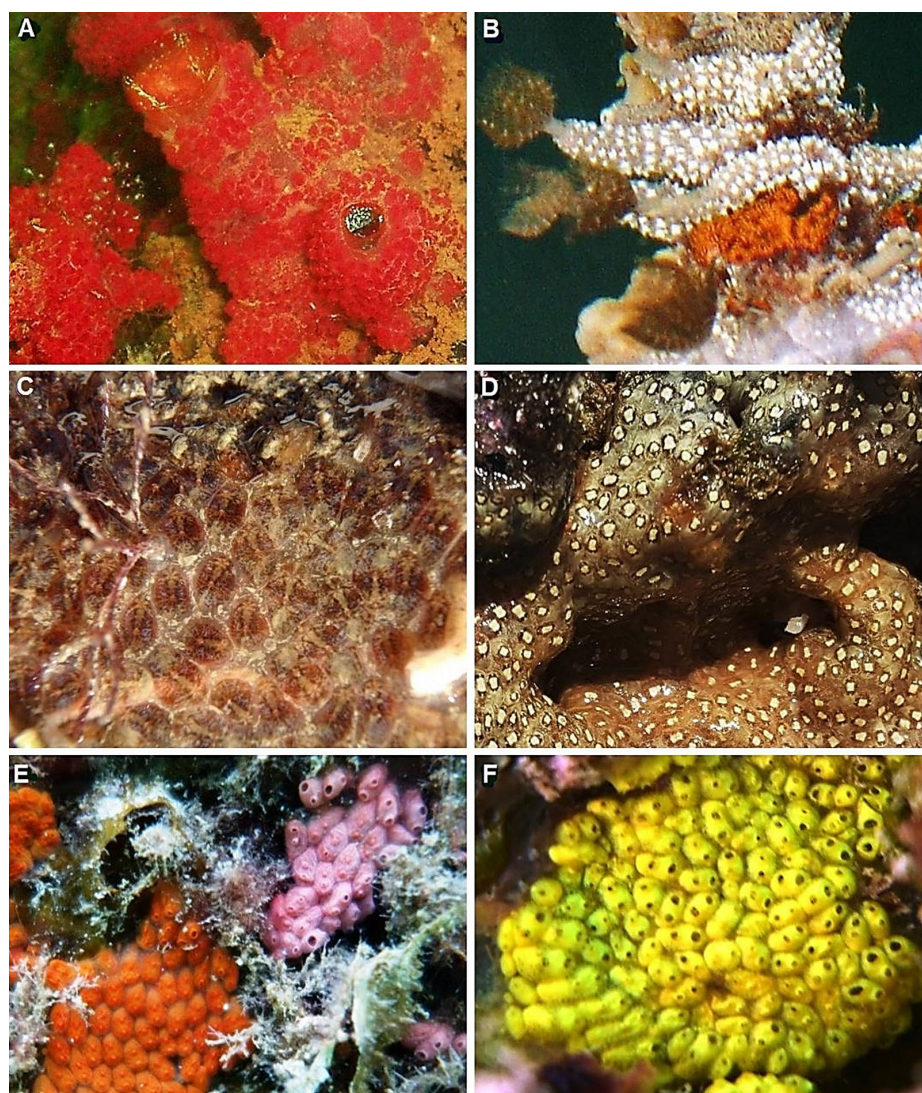


Figure 1. Colour morphs of *Symplegma brakenhielmi* living colonies in the Mediterranean Sea: A) Beirut airport with *Herdmania momus* (Photo by A.A. Ramos-Esplá); B) Monastir marina, Tunisia with *Botrylloides* cf. *nigrum* and *Styela plicata* (Photo by A.A. Ramos-Esplá); C) Fethiye, Turkey (Photo by A. Ulman); D) Kiyikislacik, Turkey (Photo by M.E. Çınar); E) wreck in front of Monastir (Photo by Y.R. Sghaier); F) Birzebbuga, Malta (Photo by A. Deidun).

Gynandrocarpa quadricornulis: Sluiter (1904 p. 95); *G. similis*: Sluiter (1904, p. 97). *Symplegma viride* f. *brakenhielmi* Michaelsen (1918a, p. 105; Kott 1952, p. 252); *S. viride* f. *stuhlmanni*: Michaelsen (1918b, p. 39); *S. viride* var. *brakenhielmi* Van Name (1921, p. 407); *S. oceania* Tokioka (1961, p. 114); *S. stuhlmanni*: Kott (1998, p. 202); *S. brakenhielmi*: Monniot (1983 p. 429); part *S. viride* (Van Name 1945, p. 232).

Material examined: See Table S1.

Description: Crusted and thin colonies, 1–2 mm thickness and up to 12 cm in diameter. Colonies were attached to ascidians, rock, seagrasses, mussels, sabellids, ropes and other artificial structures. Living colonies were light-coloured (red, rose, yellow, orange, rose, brown, white, grey; Figure 1), that turned greyish or yellowish upon fixation. The oval zooids were embedded by the tunic, depressed dorso-ventrally (rarely erect), close together, and randomly arranged without forming apparent systems. They represented different sizes (max. 3 mm), the youngest intercalated between the adults.

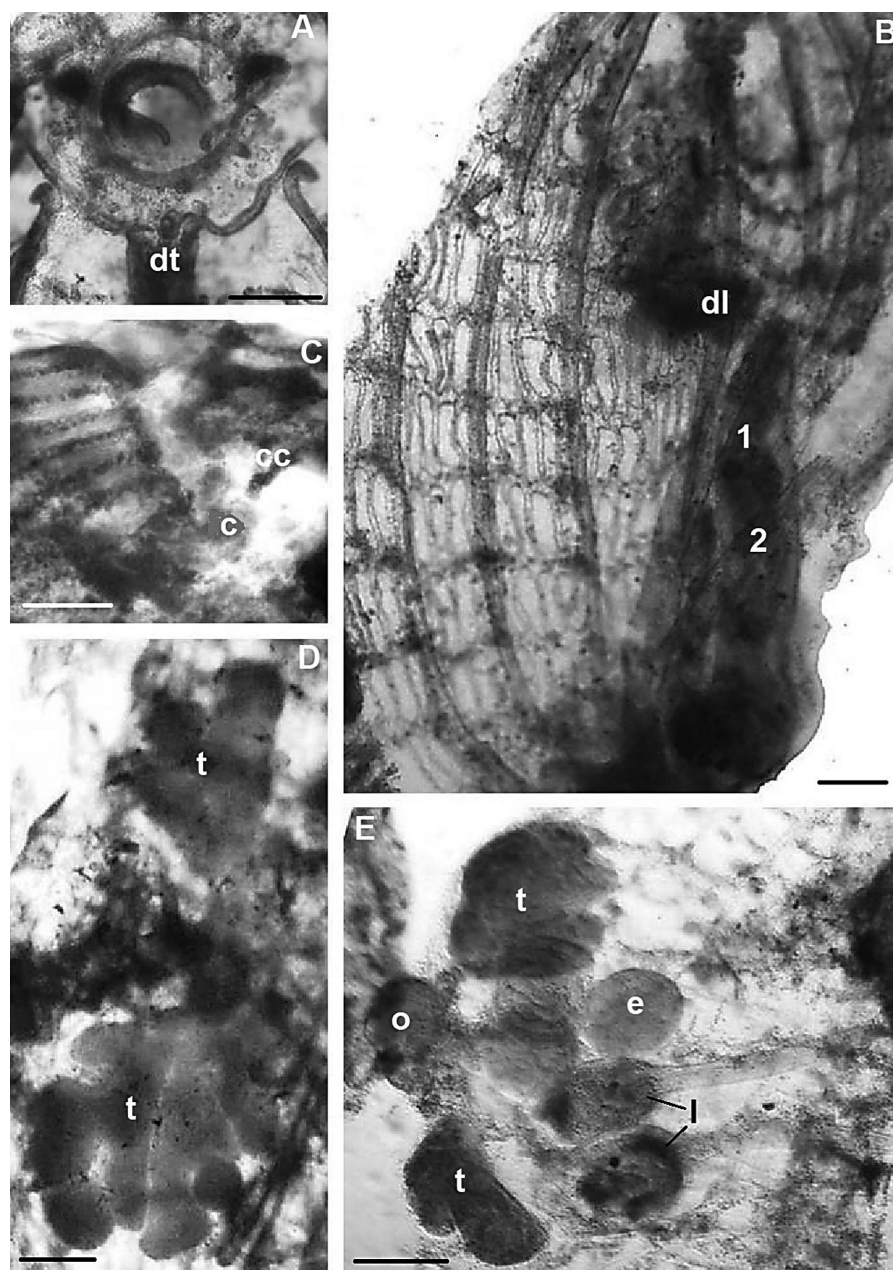


Figure 2. *Symplegma brakenhielmi*: A) peripharyngeal area with buccal tentacles and dorsal tubercle (dt); B) stomach, pyloric caecum (c) and two caecum connections (cc) with the intestine; C) branchial sac with the longitudinal vessels 1 and 2 (left side) joining the dorsal lamina (dl); D) deeply lobed testes (t); E) slightly lobed testes (t), ovary (o), embryos (e) and larvae (l) with photolith (D, zooids from Malta; E, zooids from Tunisia). Scale bars: 100 μ m (A, C, D, E), 200 μ m (B). Photos by A.A. Ramos-Esplá.

The thin test was rather tough in consistency and was transparent, allowing us to observe a “reticulated” pigmentation of the living zooids that concentrated in the branchial sac and the gut, although some colonies from Tunisia and Malta had a more opaque tunic. The siphons were similar, close and tubular and did not have coloured rings or a band around or between them. The thin and transparent mantle allowed the observation of the branchial sac and digestive tract. There were between 12 and 18 buccal simple tentacles, arranged in three orders, of which 6–9 were large and long and the others smaller (Figure 2A). The peribranchial area was bordered

by a narrow velum in a V-shape, where the oval dorsal tubercle, with a small aperture inside, was located (Figure 2A); and the dorsal lamina was smooth. There were very small atrial tentacles.

Branchial sac was without folds, with four longitudinal vessels on each side of the body, with the first two on the left being incomplete and joining the dorsal lamina at the level of 6th–7th rows of stigmata (Figure 2B). In mature zooids, about 11–13 rows of stigmata on the left and 10–12 rows on the right were present, separated by transverse vessels of uniform size, which do not meet the medium dorsal vessel exactly opposite each other. The number of stigmata per half row was between 22 and 25, and there were usually from 4 to 5 stigmata between the internal longitudinal vessels, except next to the dorsal lamina, where 6–8 stigmata were present. The stigmata formula at the 5th row on the right side was the following: E 4-5 v 4 v 4 v 4 v 6-8 DL.

The gut occupied a third of the left part of the body and formed an open loop. The oesophagus was narrow and curved followed by a cylindrical or globular stomach. The stomach had between 9 and 13 well-marked longitudinal folds, and a short, curved and stout caecum, united by two connections to the intestine (Figure 2C). The rectum bent forward and ended in a non-lobed anus at the 5th–6th row of stigmata.

One hermaphrodite gonad was located on each side, with two lobed testes and an ovary with 1–4 ovules of different sizes (Figure 2D, E). Male and female gonads were present at the same time. The testes had 2–6 lobules, little or deeply divided, and the common spermiduct was narrow with variable length. In a particular colony (station MT-2), incubated eggs and 3–5 free larvae were concomitantly observed. The larvae presented a single organ (photolith) and three sharp papillae radially ranged (Figure 2E), with a length of 0.9 mm, corresponding to a length of 320 µm up to the trunk.

Biology and ecology (Mediterranean Sea): The colonies sampled in summer (July–September) and early autumn (October) showed fully developed gonads and free fertilized ovules; the colonies from Monastir (Tunisia) sampled at the end of August contained larvae.

Symplegma brakenhielmi colonised both natural (infralittoral rocky bottoms, seagrass meadows and caves) and artificial habitats (harbours, marinas), between 0.5 and 17 m of depth. It was observed attached to many different substrates: rocks, seagrasses (*Cymodocea nodosa*, *Halophila stipulacea*, known to occur in the area; Sghaier et al. 2011), ropes, pillars, ship hulls and on living invertebrates (i.e., other ascidians, bivalves, sabellids, hydrozoans).

Global distribution: Table 1 shows the distribution of the *Symplegma* spp. throughout the world. *Symplegma brakenhielmi* represents the most widely-distributed species, both in tropical (Atlantic and Indo-Pacific) and in warm-temperate waters (NE, NW and SW Atlantic; NW Pacific; Mediterranean Sea; and Australasia).

Table 1. Global distribution of the *Symplegma* spp. (regions and provinces, according to Spalding et al. 2007). Recorded as: (a) part. *S. viride*; (b) *S. viride* f. *brakenhielmi*; (c) *S. brakenhielmi*; (d) *Diandrocarpa botrylloids*; (e) *Diandrocarpa brakenhielmi* f. *tipica*; (f) *S. viride*; (g) *Diandrocarpa brakenhielmi* f. *stuhmanni*; (h) *S. brakenhielmi* v. *ceylomica*; (i) *S. viride* f. *stuhmanni*. (j) *S. oceanica*; (k) *Gynandrocarpa quadricornulis*; (m) *G. similis*; (n) *S. stuhmanni*; (o) *S. reptans*; (p) *S. aff. viride*.

Realm/province	<i>S. viride</i>	<i>S. brakenhielmi</i>	<i>S. rubra</i>	<i>S. bahraini</i>	<i>S. reptans</i>	Other <i>Symplegma</i> spp.
Temperate N Atlantic (Mediterranean Sea)	–	See Table 2	–	–	–	
Cold Temperate NW Pacific (Japan Sea)	–	–	–	–	14, 51	<i>S. japonica</i> : 24, 51
Warm Temp. NW Atlantic	8, 9 ^a , 11 ^a , 31 ^a , 74	8 ^b , 9 ^a , 11 ^a , 31 ^a	74			
Warm Temperate NW Pacific	–	61 ^j	–	–	14, 51	<i>S. connectans</i> : 14, 50, 53 <i>S. japonica</i> : 24, 50, 51
Warm Temperate NE Pacific	–	–	–	–	60 ^c , 71, 82	–
Tropical W Atlantic	1, 2 ^d , 9 ^a , 10 ^a , 11 ^a , 22 ^a , 31 ^a , 33, 39 ^a , 42, 43 ^a	3 ^e , 8 ^b , 9 ^a , 10 ^a , 11 ^a , 22 ^a , 38 ^f , 39 ^a , 42, 43 ^a , 54, 64, 67, 69, 77, 79, 86	33, 42, 54, 64, 67, 69, 77, 86	–	–	–
Tropical E Atlantic	–	13 ^f , 15 ^f , 17 ^f , 30 ^f , 46 ^f	–	–	–	–
W Indo-Pacific	–	3 ^g , 5 ^h , 6 ⁱ , 7 ⁱ , 18 ^f , 19 ^f , 26 ^f , 28 ^f , 45, 49 ^f , 57, 84 ^j	58, 68	57, 63, 65 [?] , 68	–	<i>S. zebra</i> : 68
Central Indo-Pacific	84	4 ^k , 4 ^m , 7 ^b , 16 ^b , 21 ^j , 25 ^f , 27 ^f , 35 ^f , 36 ^p , 40 ^j , 41 ^j , 44, 48 ^j , 59 ⁿ , 66, 70, 72, 85	66, 72	72	41, 44, 85	<i>S. alterna</i> : 48 <i>S. teruakii</i> : 44 ^o , 72
E Indo-Pacific	–	47 ^j , 56 ^j , 63 ^j , 83 ^r	–	–	63	–
Tropical E Pacific	–	80	80	–	82	–
Warm Temperate SW Atlantic	–	20 ^a , 23 ^a , 29 ^a , 52, 55, 67, 75, 76, 78, 81	20 ^a , 23 ^a , 29 ^a , 55, 62, 67, 76, 81	–	–	–
Temperate Australasia	–	12 ^b , 16 ^b , 37 ^f , 44 ^j , 59 ⁿ	–	–	–	<i>S. arenosa</i> : 32, 44, 59, 73

References: (1) Herdman 1886; (2) Van Name 1902; (3) Michaelsen 1904; (4) Sluiter 1904; (5) Herdman 1906; (6) Michaelsen 1918a; (7) Michaelsen 1918b; (8) Van Name 1921; (9) Van Name 1930; (10) Berrill 1932; (11) Van Name 1945; (12) Hartmeyer and Michaelsen 1928; (13) Pérès 1949; (14) Tokioka 1949; (15) Pérès 1951; (16) Kott 1952; (17) Millar 1953; (18) Millar 1955; (19) Millar 1956; (20) Millar 1958; (21) Tokioka 1961; (22) Millar 1962; (23) Rodrigues 1962; (24) Tokioka 1962; (25) Kott 1964; (26) Plante and Vasseur 1966; (27) Tokioka 1967; (28) Vasseur 1967; (29) Costa 1969; (30) Monniot 1969; (31) Van der Sloot 1969; (32) Kott 1972; (33) Monniot 1972; (34) Kott 1975; (35) Millar 1975; (36) Tokioka and Nishikawa 1975; (37) Kott 1976; (38) Millar 1977; (39) Plough 1978; (40) Kott 1981; (41) Kott and Goodbody 1982; (42) Monniot 1983; (43) Goodbody 1984; (44) Kott 1985; (45) Renganathan 1985; (46) Lafargue and Wahl 1986; (47) Monniot and Monniot 1987; (48) Monniot 1988; (49) Lafargue and Vasseur 1989; (50) Rho and Lee 1989; (51) Nishikawa 1991; (52) Rocha 1991; (53) Rho and Lee 1991; (54) Goodbody 1993; (55) Rodrigues and Rocha 1993; (56) Abbott et al. 1997; (57) Monniot and Monniot 1997a; (58) Monniot and Monniot 1997b; (59) Kott 1998; (60) Lambert and Lambert 1998; (61) Rho and Park 1998; (62) Rodrigues et al. 1998; (63) Coles et al. 1999; (64) Goodbody 2000; (65) Monniot et al. 2001; (66) Monniot and Monniot 2001; (67) Lotufo 2002; (68) Monniot 2002; (69) Goodbody 2003; (70) Lambert 2003; (71) Lambert and Lambert 2003; (72) Kott 2004; (73) Kott 2005; (74) Lambert et al. 2005; (75) Rocha and Faria 2005; (76) Rocha and Costa 2005; (77) Rocha et al. 2005a; (78) Rocha et al. 2005b; (79) Rocha et al. 2010; (80) Carman et al. 2011; (81) Rocha et al. 2011; (82) Tovar-Hernández 2012; (83) Carlton and Eldredge 2015; (84) Abdul Jaffar and Tamilselvi 2016; (85) Lee et al. 2016.

Mediterranean Sea distribution: Table 2 and Figure 3 show the spread of *S. brakenhielmi* through the Levantine, Ionian, Aegean and Tyrrhenian Seas, with the first recorded year.

Discussion

Determination of Symplegma in the Mediterranean Sea

The genus *Symplegma* was created by Herdman in 1886 (p. 144) and was initially represented by the single species *S. viride*. Michaelsen (1904, p. 50) regarded this genus as *nomen dubium* due to Herdman's incomplete description and subsequently replaced it with the genus *Diandrocarpa* (Van

Table 2. Records of *Symplegma brakenhielmi* (Michaelsen, 1904) in the Mediterranean Sea by locality, year of the first record and references. Habitat: (A) artificial; (N) natural. Colour (living colonies): (b) brown; (c) cream; (g) greenish-gray; (o) orange; (p) pink; (r) red; (w) white; (y) yellow. (Id) identification by, (obs.) observed by (coll.) collected by: (AD) Alain Deidun; (AR) Alfonso Ramos-Esplá; (AU) Aylin Ulman; (GB) Ghazi Bitar; (JF) Jasmine Ferrario; (MEÇ) Melih E. Çinar; (YRS) Yassine R. Sghaier.

Region	Zone	Locality	Year	Habitat	Colour	Source*	Observations
Levantine Sea	Israel	Cesarea	1951	–	–	1	as <i>S. viride</i>
“	“	Haifa Bay	1975	–	–	8	det. R.H. Millar as <i>S. viride</i>
“	“	Hadera, Achziv, Palmahim	2005–2006	A,N	r	8	–
“	Egypt	Alexandria, Damietta	1987–1988	–	–	9	–
“	“	Porto Marina (Alamein)	2013	A	r	present work	coll. YRS (Id. AR)
“	Lebanon	Dalia (Beirut)	1991–1992	A	r	unpublished	Id. F. Monniot
“	“	Several locat. (Beirut, Tripoli, Batroun etc.)	1992–2016	A,N	r,p	2,3,4,6,7,10	coll. GB (Id. AR); CEDRE
“	Turkey	Yumurtalik	2005	A	r	5,7	–
“	“	Marmaris, Fethiye, Finike	2008–2016	A	b,w,r,p	12,14	coll. MEÇ, coll. AU (Id. AR)
“	Cyprus	Famagusta, Larnaca	2016	A	r	11,14	coll. AU (Id. AR)
Aegean Sea	Turkey	Akbük	2016	A	g	13	–
“	“	Kiyikislacik	2016	A	b	present work	coll. MEÇ (Id. AR)
“	Greece	Heraklion, Agios Nicholas (Crete)	2015	A	b,g	14	coll. AU
“	“	Mandraki port (Rhodes)	2016	A	w	14	coll. AU
Ionian Sea	Tunisia	Cap Monastir marina	2008, 2014	A,N	w,r	present work	coll. YRS (Id. AR)
“	“	Ship wreck front to Monastir	2017	A	w,r,o,y,p,c	present work	coll. YRS (Id. AR)
“	“	El Kantaoui port	2015	A	r	unpublished	coll. YRS
“	“	Goulette Canal	2015	A	r	unpublished	coll. YRS
“	Malta	Birzebbuga (Marsaxlokk)	2018	A	y	present work	coll. AD, JF (Id. AR)
“	Italy	Taranto	2016	A	w,r,y	15	–
Tyrrhenian Sea	Italy	Palermo	2016	A	r	12,14	coll. AU (Id. AR)
“	“	N-E Sardinian coasts	2014, 2018	A	w,r,y	15	–

* (1) Pérès (1958a); (2) Bitar and Kouli-Bitar (2001); (3) Abboud-Abi Saab et al. (2003); (4) Izquierdo-Muñoz et al. (2006); (5) Çinar et al. (2006); (6) Bitar et al. (2007); (7) Izquierdo-Muñoz et al. (2009); (8) Shenkar and Loya (2009); (9) Halim and Abdel-Messeih (2016); (10) RAC/SPA-UNEP/MAP (2015); (11) Savva and Kleitou (2017); (12) Ulman et al. (2017); (13) Aydin-Onen (2018); (14) Ulman (2018); (15) Mastrototaro et al. (2019).



Figure 3. Distribution of *Symplegma brakenhielmi* (red quadrats, new records are highlighted with an asterisk) in the Mediterranean Sea and Red Sea; year of first record (see references in Table 2), surface current direction (Hamad et al. 2005) and boundaries of the Mediterranean sub-basins (dashed lines) are also represented.

Name, 1902) containing the single species *D. brakenhielmi*. However, Van Name (1921, 1930, 1945) considered both species synonymous, giving priority to *S. viride*, stating that *S. brakenhielmi* cannot even be maintained as a subspecies of *S. viride*. This categorical statement was supported by subsequent authors until the 1980s, when Monniot (1983) established the differences between the aforementioned two species (*S. viride* and *S. brakenhielmi*), together with *S. rubra*, in Caribbean waters. Therefore, many pre-1983 publications on *Symplegma* in the Western and Eastern Atlantic, as well as in the Indian Ocean, adopted Van Name's description (1945) and considered these congeneric species as a single species: *S. viride*. Thus, one can infer that the first records of *Symplegma* (as *S. viride*; Herdman 1886) in the Suez Canal and Mediterranean Sea (Harant 1927; Pérès 1958a, b; Steiniz 1967; Por 1978; Koukouras et al. 1995), and Tropical Western Africa (Pérès 1949, 1951; Millar 1953; Monniot 1969; Lafargue and Wahl 1986) correspond to *S. brakenhielmi*.

Symplegma brakenhielmi, *S. rubra* and *S. bahraini* present lobed testes in contrast to *S. viride* and *S. reptans* without lobed testes. However, in *S. brakenhielmi*, the tunic is transparent in living colonies with the pigments localised in the blood cells, thus imparting a reticulated aspect due to the concentration in the branchial sac and gut (*S. rubra* and *S. bahraini* have opaque tunics). Additionally, *S. brakenhielmi* does not have the characteristic red rings that encircle both siphons (a characteristic of *S. rubra*); although Herdman (1906) and Tokioka (1961) do indicate the presence of red circles around the siphons in *S. brakenhielmi* var. *ceylonica* and *S. oecania* (a synonym of *S. brakenhielmi*), respectively. Other characters, such as the number of testes lobes and the division between them (more numerous and deeper in *S. rubra* and *S. bahraini*) as well as the length and width of the common spermiduct are less diagnostic (Monniot 2002; Couto 2003). The simultaneous presence of testes and ovules in *S. brakenhielmi* and *S. bahraini* separates them from *S. rubra* (Monniot 1983; Monniot and Monniot 1997a), but this is not applicable for immature zooids (Monniot et al. 2001). Rocha and Costa (2005) included the following other characters to distinguish *S. brakenhielmi* from *S. rubra*: the swollen dorsal tubercle of the former, with a small circular aperture inside; two incomplete dorsal left longitudinal vessels, reaching the dorsal lamina at the level of the 4th and 7th rows of stigmata; and only two tissue connections linking the caecum with the intestine. These characteristics have all been observed in the zooids of samples originating from the Mediterranean, and a molecular study conducted by Mastrototaro et al. (2019) corroborates the presence of *S. brakenhielmi* in the basin.

It has to be noticed that the pigmentation of the tunic and the deeply divided testicular follicles from the Maltese and Tunisian (i.e. Monastir) specimens resemble *S. bahraini* (Monniot and Monniot 1997a; Kott 2004). However, Monniot and Monniot (1987: 10) found colonies of *S. oecania*

(synonymy of *S. brakenhielmi*) with a pigmented tunic; and Tokioka (1961, Figure 7) drew deeply divided testicular follicles for the same species. The presence of larvae in the Tunisian colonies with a single sensory organ (Millar 1953; Kott 1985; Mastrototaro et al. 2019) suggests that these colonies are more closely related to *S. brakenhielmi*, since *S. bahraini* has two sensory organs (Monniot and Monniot 1997a). Furthermore, the remaining morphological characters fall within the range of *S. brakenhielmi* and its synonyms (Monniot and Monniot 1997a; Monniot 2002; Kott 2004), such as its stomach folds, rows of stigmata, stigmata per row, pyloric caecum and its links with the intestine; longitudinal vessels on the left join the dorsal lamina, and the simultaneous presence of functional male and female gonads.

In the present study some colonies sampled in summer and autumn showed fully developed gonads or contained larvae. Larvae had also been recorded in June in Israel (Levantine Sea) by Shenkar and Loya (2009); and in July in North-eastern Sardinia (Tyrrhenian Sea) by Mastrototaro et al. (2019). Generally, data on reproductive period is scarce and scattered, e.g. from January to May in the Tropical Eastern Atlantic (Senegal: Pérès 1949; Monniot 1969; Ghana: Millar 1953), as well as immature colonies in January (Pérès 1951); in May in the Southern Indian Ocean (Mauritius: Vasseur 1967); from July to October in the Northern Indian Ocean (S-India: Renganathan 1985; Bahrain: Monniot and Monniot 1997a); and in November in the Tropical Pacific (W-Australia: Kott 1985).

In conclusion, morphological studies on different color morphs (red, yellow, white, brown, pink) with transparent and opaque tunics led us to the same species in the Mediterranean Sea: *S. brakenhielmi*. In this regard, Mastrototaro et al. (2019) have not found significant morphological or genetic differences between red and yellow colonies of *S. brakenhielmi* from the Western (Sardinia) and Central (Taranto) Mediterranean Sea, thus highlighting the possible synonymy between *S. brakenhielmi* and *S. rubra* by Automatic Barcode Gap Discovery (ABGD) species delimitation analysis. However, to confirm this, it is necessary to perform genetic analysis of our specimens and compare them with *S. bahraini* and *S. rubra*.

Possible causes of spread in the Mediterranean Sea: routes and vectors

Michaelsen (1918b) recorded *S. brakenhielmi* (as *S. viride* f. *stuhlmanni*) in the Gulf of Suez (Red Sea) in 1914. Later in 1924, Harant (1927) found it in the Suez Canal at El Katera, 46 km away from Port Said. Pérès (1958a) reported the species (as *S. viride*) in 1951 from the Mediterranean coast of Israel, together with *Ascidia cannelata* Oken, 1920, *Phallusia nigra* Savigny, 1816 and *Hermania momus* Savigny, 1816, solitary ascidians of Indo-Pacific origin. The same author (Pérès 1958b) also argued how these new findings were probably recent introductions from the Red Sea via the Suez Canal, since he had only found them in Israel, and not in Syria, Greece or Turkey.

In the Levantine Sea, *S. brakenhielmi* has progressively spread northwards, following the trajectory of the prevailing surface coastal current (Figure 3; Hamad et al. 2005), and has become a common species on both artificial and natural habitats in this area (Bitar and Kouli-Bitar 2001; Çinar et al. 2006; Izquierdo-Muñoz et al. 2006, 2009; Bitar et al. 2007; Shenkar and Loya 2009). In Cyprus it was found on artificial substrata in harbours in 2016 (Savva and Kleitou 2017; Ulman 2018; Ulman et al. 2019), although a study conducted in the same country in Larnaca Bay in June 2007 (UNEP-MAP RAC/SPA 2007) did not find the species; this may suggest a recent introduction mediated by shipping. Regarding the westward spread further along the coasts of Egypt, Halim and Abdel Messeih (2016) recorded the species in Alexandria in 1988–89 and in El Alamein, west of Alexandria, in 2013 (YRS *pers. obs.*, present study), both within ports. All living colonies observed were the reddish or pink colour morphs (Figure 1A).

The recent appearance of *S. brakenhielmi* along the Aegean coasts of Turkey (in 2015–2016 at Kiyikislacik and Akbük; MEÇ *pers. obs.* and Aydin-Onen 2018) and Greece (Rhodes and Heraklion; Ulman 2018; Ulman et al. 2019) might suggest a further expansion of the species within the Aegean Sea through the Eastern Mediterranean surface current. However, these observed colonies had white, brown and greenish-grey morphs (not red; Figure 1C–D) and were found in harbours and rocks near fish-farm cages, which could suggest either aquaculture and/or shipping as the main introduction vectors.

In the Ionian Sea, the first record was made in October 2008 (Monastir Marina, Tunisia; AR *pers. obs.*), followed by new records in October 2014 (present study). The species was observed on rocks and seagrass meadows (*Cymodocea nodosa* and *Halophila stipulacea*) around the harbour with white and red colour morphs. Studies carried out in 2005 and 2006 in Hammamet, 70 km north of Monastir (Chabbi et al. 2010), and 2009–2010 in the Gulf of Gabes (Zarzis and Sfax harbours, Ramos-Esplá et al. 2011), did not find this species. In December 2014, the merchant-ship “Rochelle” sailing from Ghana to Turkey was stranded near the Kuriat Islands in front of Monastir, and was colonized by abundant colonies of *S. brakenhielmi* with different colours (red, pink, yellow, orange, cream, white; Figure 1) on the hull. Later, the species was recorded in other Tunisian localities further north (YRS *pers. obs.*), including the port of El Kantaoui (October 2015) and Goulette Canal (November 2015), all with red color morph. Monastir is an important marina for boat traffic, thus possibly representing a propagule seeding hub for adjacent ports. International maritime traffic by commercial vessels could be considered the most likely vector of introduction of *S. brakenhielmi*, e.g. specifically for Marsaxlokk (Malta), which represents one of the main cargo trans-shipment ports of the Mediterranean Sea; recreational boats could also be a vector (Ulman et al. 2017; Ulman 2018; Mastrototaro et al. 2019). *Symplegma brakenhielmi* was

frequently recorded within several sampled marinas, and it was also found on recreational boat hulls in Turkey (Ulman et al. 2017), suggesting that recreational boating may be an important secondary vector in its rapid expansion through the intensely sailed Mediterranean Sea (Cappato 2011).

As previously mentioned, together with *S. brakenhielmi*, Pérès (1958a) also identified three other solitary Lessepsian ascidians: *A. cannelata*, *P. nigra* and *H. momus*. While the former ascidian remains localised to the south-eastern portion of the Levantine Sea (Izquierdo-Muñoz et al. 2009; Shenkar and Loya 2009), *P. nigra* and *H. momus* have formed proliferating populations further northward, following the same colonization dispersal pattern promoted by surface currents through the Levantine and Aegean Seas (Çinar et al. 2006; Kondylatos et al. 2010; Koutsogiannopoulos et al. 2012; Gerovasileiou and Issaris 2014; Kondylatos and Corsini-Foka 2017). At present, only *H. momus* has reached the Ionian Sea in Marsaxlokk, Malta (Evans et al. 2013), which is very close to the sampling site of *S. brakenhielmi* recorded in this study and was most likely introduced through maritime traffic.

Therefore, the colonial *S. brakenhielmi* appears to be spreading faster than these three solitary ascidians. The possible causes of this postulated expansion could be traced to the characteristics of its colonial strategy and larval development (Millar 1971). The rapid growth (as encrusting colonies) and short life cycle of this species are, in fact, competitive strategies for space (Jackson 1977). *Symplegma brakenhielmi* exhibits two-dimensional growth, and the larva develops inside the zooids (Berrill 1940), which is advantageous for the colonization of artificial structures within fouling communities and for growth as an epibiont (Green et al. 1983; Ramos-Esplá and Ros 1990; Rocha 1991; Dijkstra et al. 2007), whereas *P. nigra* and *H. momus* are oviparous ascidians, with less-specialised larva (Millar 1971).

Ascidians are one of the most frequent NIS in artificial habitats due to their rapid spread and population outbreaks (Lambert 2007; Zhan et al. 2015). Generally, ascidians can be associated with multiple vectors of introduction, including ballast water, rafting, hull fouling and aquaculture (Zhan et al. 2015). While the role of ballast water in the transport of ascidians remains unclear, several studies provide evidence for the colonisation success of ascidians on recreational boat hulls as biofouling components (e.g. Darbyson et al. 2009; Lambert 2002; Ulman et al. 2017), rafting colonies living attached to seagrass leaves (Worcester 1994), and in association with shell-fish aquaculture facilities (e.g. Davis et al. 2007; Ordóñez et al. 2015). Among the *Symplegma* genus, the three species recorded in two or more distant biogeographic regions (i.e., *S. brakenhielmi*, *S. rubra* and *S. reptans*; Table 1) are likely to have been associated with the biofouling and aquaculture vectors of introduction and spread (Rocha et al. 2009; Carman et al. 2011; Mastrototaro et al. 2019; Ulman et al. 2019). In addition, colonies of *S. rubra* have also been observed on free-floating

algae and other drifting materials (Dias et al. 2006), thus could have been transported by rafting. Similarly, *Symplegma* species recorded in closer biogeographic regions (i.e. *S. viride*, *S. bahraini* and *S. japonica*; Table 1) may have locally spread through shipping or by natural means (Tamilselvi et al. 2011; Zhan et al. 2015).

Currently, regulations addressing the control of NIS only relate to aquaculture (EC 2007) and ballast water (Ballast Water Management Convention of the International Maritime Organization, IMO) vectors, while management of the biofouling vector is still unregulated and insufficiently assessed in the Mediterranean Sea. Specific management guidelines for this vector were previously formulated (IMO 2011), and an ad-hoc project (GEF-UNDP-IMO GloFouling Project) was launched in December 2018 in order to address the management of biofouling. The continuous monitoring programmes in high-risk areas of NIS introduction (e.g. ports and aquaculture facilities) and the use of preventive measures to limit NIS spread are recommended best practices against marine bioinvasions (Lehtiniemi et al. 2015). The Mediterranean Sea brings together many national jurisdictions, placing the onus for the effective management of new introductions and their related vectors on a basin-wide early detection and warning system. Finally, the dissemination of taxonomic knowledge and the elucidation of ambiguous taxonomy are essential for an accurate species identification, particularly urgent for the class Ascidiacea (Shenkar and Swalla 2011; Zhan et al. 2015).

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Supplementary material

The following supplementary material is available for this article:

Table S1. Sampling information on the analysed specimens.

This material is available as part of online article from:

http://www.reabic.net/aquaticinvasions/2020/Supplements/AI_2020_Ramos-Espla_etal_SupplementaryTable.xlsx